Arabidopsis thaliana amiRNA library

CONTENTS

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Description</td>
<td>1</td>
</tr>
<tr>
<td>Background and Design Information</td>
<td>1</td>
</tr>
<tr>
<td>Vector Information</td>
<td>1</td>
</tr>
<tr>
<td>Vector Map - pAmiR</td>
<td>3</td>
</tr>
<tr>
<td>Antibiotic Resistance</td>
<td>3</td>
</tr>
<tr>
<td>Quality Control</td>
<td>3</td>
</tr>
<tr>
<td>Protocol I - Replication</td>
<td>4</td>
</tr>
<tr>
<td>Protocol II - Plasmid Preparation</td>
<td>5</td>
</tr>
<tr>
<td>Protocol III - Restriction Digest</td>
<td>6</td>
</tr>
<tr>
<td>Protocol IV - Agrobacterium Electroporation</td>
<td>6</td>
</tr>
<tr>
<td>Protocol V - Arabidopsis Transformation</td>
<td>7</td>
</tr>
<tr>
<td>Protocol VI - Selection of Primary Transformants</td>
<td>8</td>
</tr>
<tr>
<td>Related Reagents</td>
<td>8</td>
</tr>
<tr>
<td>FAQs</td>
<td>9</td>
</tr>
<tr>
<td>Troubleshooting</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Licensing Information</td>
<td>10</td>
</tr>
</tbody>
</table>

PRODUCT DESCRIPTION

The Arabidopsis thaliana amiRNA library is a collection of artificial microRNAs (amiRNAs) developed by Dr. Greg Hannon at Cold Spring Harbor Laboratories (CSHL) in collaboration with Drs. Rob Martienssen and Dick McCombie at CSHL and the Weigel lab at the Max Planck Institute for Developmental Biology. This resource is being constructed at CSHL and will eventually target 22,000 Arabidopsis genes with three constructs per gene.

This collection is the first genome-wide resource for plant RNAi and provides an excellent tool for gene silencing in Arabidopsis due to the specificity and predictability of the effects of plant amiRNA.

BACKGROUND AND DESIGN INFORMATION

The amiRNA design is based on the work of Detlof Wiegel and colleagues at the Max Planck Institute for Developmental Biology. amiRNA constructs in the Arabidopsis thaliana amiRNA library are expressed in a miR319a backbone, engineered with unique EcoRI and HindIII restriction sites and cloned into pAmiR, a modified version of the pGREEN vector. Design elements include a uridine at position 1, and if possible, an adenine at position 10 both of which are overrepresented among natural plant miRNAs and highly efficient siRNAs (Mallory, Reinhart et al. 2004; Reynolds, Leake et al. 2004). The amiRNAs also display 5’ instability relative to their miRNA, so that the correct sequence would be incorporated into RISC. To reduce the likelihood that an amiRNA would act as primer for RNA-dependent RNA polymerases, and thereby trigger secondary RNAi, between one and three mismatches to the target genes were introduced in the 3’ part of the amiRNAs.

These amiRNAs have been shown to be effectively produced from their precursors and processed as the intended 21mer (Schwab, Palatnik et al. 2005; Schwab, Ossowski et al.)
amiRNAs are processed by DICER-LIKE1 (DCL1) in plants producing a stable RNA that is then incorporated into the silencing complex RISC triggering the transcript cleavage and degradation of the target gene. Extensive validation of the amiRNA design in producing phenotypes, decreasing RNA levels and altering the expression of downstream target genes is detailed in Schwab, Palatnik et al. 2005; and Schwab, Ossowski et al. 2006.

VECTOR INFORMATION
To enable the efficient and convenient use of artificial microRNAs (amiRNAs), synthesized hairpins were cloned into a binary plasmid conferring strong and constitutive expression that can directly serve for plant transformation. For that purpose the 35S promoter together with a 3’ocs (Octopine synthase terminator) terminator were transferred into a widely used pGreenII (http://www.pgreen.ac.uk) binary variant that confers Basta® resistance to transgenic plants called pAmiR™, (Figures 1-2, and Table 1). Promoter and terminator regions encompass genomic sequences surrounding and containing the miR319a hairpin, which is the template for amiRNA construction. Single base-pair substitutions that generate unique restriction sites have been engineered to separate the hairpin from flanking sequences (EcoRI and HindIII, see Figure 2), such that newly synthesized amiRNA hairpins can be embedded into the miR319a genomic context. These sites do not affect the silencing potential of the hairpin precursor, i.e. they do not interfere with downstream phenotypes that are caused by small RNA accumulation (both when using the miR319a backbone alone and also when an amiRNA is engineered). Gateway® AttB as well as conventional restriction sites 5’ and 3’ of the complete amiRNA precursor allow the transfer to other plasmids, such as those with more restrictively active promoters.

Figure 1. pAmiR vector

Table 1. Features of the pAmiR™ vector

<table>
<thead>
<tr>
<th>Vector Element</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left Border</td>
<td>T-DNA boundary</td>
</tr>
<tr>
<td>35S promoter</td>
<td>Promoter for amiRNA expression in plants</td>
</tr>
<tr>
<td>AttB sites</td>
<td>Gateway® cloning sites</td>
</tr>
<tr>
<td>3’ocs</td>
<td>Octopine synthase terminator</td>
</tr>
<tr>
<td>Basta® resistance</td>
<td>Plant selection marker</td>
</tr>
<tr>
<td>pSa origin</td>
<td>Origin of replication for E.coli and Agrobacterium</td>
</tr>
<tr>
<td>Right Border</td>
<td>T-DNA boundary</td>
</tr>
<tr>
<td>Spectinomycin resistance</td>
<td>Bacterial selection marker</td>
</tr>
<tr>
<td>pUC origin</td>
<td>Origin of replication for E.coli</td>
</tr>
</tbody>
</table>
Figure 2. Detailed vector map of pAmiR\textsuperscript{TM} vector.

**ANTIBIOTIC RESISTANCE**

pAmiR\textsuperscript{TM} contains 2 antibiotic resistance markers (Table 2).

**Table 2.** Antibiotic resistances conveyed by pAmiR

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectinomycin</td>
<td>100µg/ml</td>
<td>Bacterial selection marker</td>
</tr>
<tr>
<td>Basta\textsuperscript{®}</td>
<td>variable</td>
<td>Plant selectable marker</td>
</tr>
</tbody>
</table>

**QUALITY CONTROL**

Constructs have been sequenced verified, digested for correct size (Figure 3) and tested for phage contamination.
Figure 3. Sample agarose gel of pAmiR digestion performed for quality control. Alternating lanes are undigested and digested using NotI. NotI digestion results in a 4.3kb and ~2kb band (2kb band may vary slightly with size of miRNA).

**PROTOCOL I - REPLICATION**

**Table 3. Materials for plate replication**

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-Lennox broth (low salt)</td>
<td>VWR</td>
<td>EM1.00547.0500</td>
</tr>
<tr>
<td>Peptone, granulated, 2kg - Difco</td>
<td>VWR</td>
<td>90000-368</td>
</tr>
<tr>
<td>Yeast Extract, 500g, granulated</td>
<td>VWR</td>
<td>EM1.03753.0500</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sigma</td>
<td>S-3014</td>
</tr>
<tr>
<td>Glycerol</td>
<td>VWR</td>
<td>EM-2200 or 80030-956</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>VWR</td>
<td>Calbiochem 567570</td>
</tr>
<tr>
<td>96 well microplates</td>
<td>Nunc</td>
<td>260860</td>
</tr>
<tr>
<td>Aluminum seals</td>
<td>Nunc</td>
<td>276014</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Genetix</td>
<td>X5054</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Scinomix</td>
<td>SCI-5010-OS</td>
</tr>
</tbody>
</table>

For archive replication, grow all amiRNA clones at 37°C in LB-Lennox (low salt) media plus 100µg/ml spectinomycin. Prepare media with 8% glycerol* and the appropriate antibiotics.

**Replication of plates**

Prepare target plates by dispensing ~160µl of LB-Lennox (low salt) media supplemented with 8% glycerol* and appropriate antibiotic (100µg/ml spectinomycin).

Prepare source plates:
1. Remove foil seals while the source plates are still frozen. This minimizes cross-contamination.
2. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.
Replicate:
1. Gently place a disposable replicator in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the well.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.
5. Repeat steps 1-4 until all plates have been replicated.
6. Return the source plates to the -80°C freezer.
7. Place the inoculated target plates in a 37°C incubator without shaking for 18-19 hours.

Freeze at -80°C for long term storage. Avoid long periods of storage at room temperature or higher in order to control background recombination products.

*Glycerol should be omitted from the media if you are culturing for plasmid preparation. If making copies of the constructs for long term storage at -80°C, 8% glycerol is required.

PROTOCOL II - PLASMID PREPARATION
Culture conditions for individual plasmid preparations
For plasmid preparation, grow all pAmiR™ clones at 37°C in LB broth (low salt) media plus 100µg/ml spectinomycin.

LB broth (low salt) media preparation
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-Broth-Lennox</td>
<td>10g/l</td>
</tr>
<tr>
<td>Peptone</td>
<td>10g/l</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5g/l</td>
</tr>
<tr>
<td>Salt</td>
<td>5g/l</td>
</tr>
<tr>
<td></td>
<td>Appropriate antibiotic(s) at recommended concentration(s)</td>
</tr>
</tbody>
</table>

Most plasmid mini-prep kits recommend a culture volume of 1-10ml for good yield. For pAmiR constructs, 5ml of culture can be used for one plasmid mini-prep generally producing 5-10µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNAmir of interest store at -80°C until ready to begin.
2. To prepare plasmid DNA first thaw your glycerol stock culture and pulse vortex to resuspend any E. coli that may have settled to the bottom of the tube.
3. Take a 10µl inoculum from the glycerol stock into 3-5ml of 2X-LB (low salt) with 100µg/ml spectinomycin. Return the glycerol stock(s) to -80°C.

   Note: If a larger culture volume is desired, incubate the 3-5ml culture for 8 hours at 37°C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.

4. Incubate at 37°C for 18-19 hours with vigorous shaking.
5. Pellet the 3-5ml culture and begin preparation of plasmid DNA.
6. Run 3-5µl of the plasmid DNA on a 1% agarose gel. pAmiR with miRNA is 6.4kp.
Culture conditions for 96 well bio-block plasmid preparation
Inoculate a 96 well bio-block containing 1ml per well of LB (low salt) media with 100µg/ml spectinomycin with 1µl of the culture. Incubate at 37°C with shaking (~170-200rpm). We have observed that incubation times between 18-19 hours produce good plasmid yield. For plasmid preparation, follow the protocols recommended by the plasmid isolation kit manufacturer.

Note: Open Biosystems uses the above 96 well bio-block plasmid preparation protocol in conjunction with a Qiagen Turbo kit (catalog no. 27191). We use 2 bio-blocks combined, do not perform the optional wash and elute the DNA in water.

PROTOCOL III - RESTRICTION DIGEST
The following is a sample protocol for restriction enzyme digestion using NotI for diagnostic quality control of pAmiR vectors. A digestion with NotI should result in a 4.3kb and a ~2kb band (2kb band may vary slightly with size of miRNA).

1. Using filtered pipette tips and sterile conditions add the following components, in the order stated, to a sterile PCR thin-wall tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, nuclease-free water</td>
<td>Xµl</td>
</tr>
<tr>
<td>Restriction enzyme 10X buffer</td>
<td>1µl</td>
</tr>
<tr>
<td>DNA sample (400ng) in water</td>
<td>400ng</td>
</tr>
<tr>
<td>NotI 10U (NEB)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>

PROTOCOL IV- AGROBACTERIUM ELECTROPORATION*
Electroporation of Agrobacterium bacteria

Table 4. Materials for electroporation

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appropriate Agrobacterium strain</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>ice-cold sterile 10% glycerol solution (to prepare comp cells)</td>
<td>VWR</td>
<td>EM-2200 or 80030-956</td>
</tr>
<tr>
<td>liquid nitrogen</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>Helper plasmid pSOUP</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>Electroporation equipment</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>LB-Lennox broth (low salt)</td>
<td>VWR</td>
<td>EM1.00547.0500</td>
</tr>
<tr>
<td>Peptone, granulated, 2kg - Difco</td>
<td>VWR</td>
<td>90000-368</td>
</tr>
<tr>
<td>Yeast Extract, 500g, granulated</td>
<td>VWR</td>
<td>EM1.03753.0500</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sigma</td>
<td>S-3014</td>
</tr>
<tr>
<td>Glycerol</td>
<td>VWR</td>
<td>EM-2200 or 80030-956</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>VMR</td>
<td>Calbiochem 567570</td>
</tr>
</tbody>
</table>

* Commonly used electrocompetent strains are GV3101 (rifampicin and gentamycin resistant) (Koncz and Schell 1986) and ASE (kanamycin and chloramphenicol resistant) (Fraley et al., 1985)

Preparation of electrocompetent cells
1. Grow a small overnight culture (~5ml) in LB with appropriate antibiotics (see above). Grow at 28-30°C.
2. Inoculate a 500ml culture with these cells and grow at 28-30°C for several hours until an OD<sub>600</sub> of 0.5-0.7 is reached. Start this culture early in the morning to proceed in the
afternoon, or in the evening to harvest cells in the morning. The doubling time of Agrobacteria at this temperature is 2-3h.

3. Cool cells down on ice for 15 or more minutes and proceed in ice or in the coldroom for all of the following steps.

4. Harvest cells by centrifugation at 3000g for 10 minutes at 4°C (cool rotor down before use).

5. Wash pellets twice with 1 volume pre-cooled 10% glycerol solution and spin as above.

6. Resuspend pellets in 2ml 10% glycerol and combine.

7. Prepare 40µl aliquots and freeze rapidly in liquid nitrogen.

8. Store cells at -80°C.

Electroporation
1. Thaw cells on ice (1 aliquot per plasmid)
2. Add 1µl of clean plasmid DNA and 1µl of helper plasmid DNA
3. Mix and transfer into pre-cooled electroporation cuvette
4. Electroporate using standard settings for E.coli (pulse around 5msec)
5. Immediately add 1ml of LB and transfer to a reaction tube (2ml or 15ml)
6. Incubate for 2-3 hours at 28-30°C with gentle agitation
7. Collect cells by spinning briefly at 3000g
8. Plate all cells on LB plates with appropriate antibiotics
9. Incubate for 1.5 to 3 days at 28-30°C
10. Optional: re-streak colonies on a new plate and incubate as before

PROTOCOL V- ARABIDOPSIS TRANSFORMATION*
Generating transgenic Arabidopsis thaliana plants with the amiRNA binary plasmids

Table 5. Materials for transformation

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-Lennox broth (low salt)</td>
<td>VWR</td>
<td>EM1.00547.0500</td>
</tr>
<tr>
<td>Peptone, granulated, 2kg - Difco</td>
<td>VWR</td>
<td>90000-368</td>
</tr>
<tr>
<td>Yeast Extract, 500g, granulated</td>
<td>VWR</td>
<td>EM1.03753.0500</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sigma</td>
<td>S-3014</td>
</tr>
<tr>
<td>Murashige and Skoog Salts</td>
<td>Sigma</td>
<td>various</td>
</tr>
<tr>
<td>Sucrose</td>
<td>VWR</td>
<td>various</td>
</tr>
<tr>
<td>Silwet L-77 (Lehle Seeds)</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>1x Gamborg’s B5 vitamins</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>Benzylamino purine</td>
<td>various</td>
<td>various</td>
</tr>
</tbody>
</table>

Infiltration medium
1/2x Murashige and Skoog Salts
5% sucrose
optional:
1x Gamborg’s B5 vitamins
0.044µM benzylamino purine (stock solution in DMSO)
pH to 5.7 with 1M KOH
add 50µl/L Silwet L-77
Preparation of *Arabidopsis* plants for transformation

1. Grow plants in soil such that they have sufficient space to grow (rosettes should not overlap). Chose pots that correspond in size to a container that will contain the *Agrobacterium* solution for dipping. Grow 10-50 plants per plasmid.
2. Cut the first inflorescences such that more secondary shoots are formed.
3. Grow a small liquid culture from a single *Agrobacterium* colony (3-10ml) in LB with antibiotics (12-24h). Start roughly 3 days after removal of plant inflorescences.
4. Carry out either minipreps or PCR to verify the presence of the plasmids.
5. Prepare glycerol stocks (15% final glycerol concentration) from positive cultures.
6. Re-grow the positive culture and use 200µl to 1ml for inoculation of a large 200-300ml culture. Grow this culture for 12-24h until OD$_{600}$ is >1.
7. Pellet cells by centrifugation at 3000g for 10minutes.
8. Resuspend in 200-400ml infiltration medium.
9. Transfer into a convenient container, e.g. an empty tip box.
10. Dip plant inflorescences into *Agrobacterium*-containing solution for 15-30 seconds.
11. Lay pots sideways into growth flats and cover for 1 day.
12. Remove cover and return plants to their normal growth conditions.
13. Collect seeds after ~3 weeks.

**PROTOCOL VI- SELECTION OF PRIMARY TRANSFORMANTS**

The plasmids confer Basta resistance to transgenic plants. These can be selected directly on soil.

**Table 6. Materials for transformant selection**

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td>various</td>
<td>various</td>
</tr>
<tr>
<td>Basta solution</td>
<td>Scotts Company- Finale</td>
<td>unknown</td>
</tr>
</tbody>
</table>

**Selection of primary transformants**

1. Soak soil in 1:1000 dilution of Basta.
2. Spread seeds evenly on soil.
3. Cover the tray and keep at 4°C for 2-4 days. (alternatively: transfer seeds in 15ml screw-cap tube and resuspend in 0.1% agar solution, keep at 4°C for 2-4 days, then sow)
4. Move tray into normal growth conditions.
5. After several days, spray plants with 1:1000 Basta solution.
6. Selection should be evident after 7-10 days.
7. Transplant transgenic seedlings to new pots.

*Protocols courtesy of Rebecca Schwab, CSHL*

**RELATED REAGENTS**

**Table 7. Related Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em> amiRNA individual clone</td>
<td>Open Biosystems</td>
<td>AMR4844</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> amiRNA library</td>
<td>Open Biosystems</td>
<td>AMR4933</td>
</tr>
</tbody>
</table>
**FAQS**
For answers to questions that are not addressed here, please email technical support at info@openbiosystems.com with your question, your sales order or purchase order number and the catalog number or clone ID of the construct or collection with which you are having trouble.

**Useful Websites:**
For more information on the *Arabidopsis thaliana* amiRNA library, visit http://www.openbiosystems.com/RNAi/ArabidopsisthalianaamiRNA/ and http://2010.cshl.edu/scripts/main2.pl

For more information on amiRNA design, visit: http://wmd2.weigelworld.org/cgi-bin/mirnatoools.pl?page=8#mirnaFunction

For genetic and molecular biology data for *Arabidopsis* visit: http://www.Arabidopsis.org/

**What clones are part of my collection?**
A CD containing the data for this collection will be shipped with each collection. This file contains the location and accession number for each construct in the collection. This data file can be downloaded from the *Arabidopsis thaliana* amiRNA product page: http://www.openbiosystems.com/collateral/rnai/pi/Arabidopsis_manual.pdf

**Where can I find the sequence of an individual amiRNA construct?**
If you are looking for the sequence an individual amiRµA construct, you can use the gene search. Just enter the catalog number or clone ID of your construct into the gene search on the Open Biosystems website, hit submit and then click on the query result. If you then click on the oligo ID and then click on the word “sequence” in the details grid, the hairpin sequence is listed. If you are looking for the sequence of several shRµAmir constructs, you can access this information in the data file of the collection. This data file can be downloaded from the product page: http://www.openbiosystems.com/RNAi/ArabidopsisthalianaamiRNA/

**Which antibiotic should I use?**
You should grow all *Arabidopsis thaliana* amiRNA constructs in 100µg/ml spectinomycin.

**Where can I get more information on the design of the amiRNAs in this collection?**
See the reference (Schwab, Palatnik et al. 2005; Schwab, Ossowski et al. 2006).

**What if I want to design my own amiRNA?**
There is a design tool called the Web MicroRNA Designer for use with *Arabidopsis* amiRNA design at the following link: http://wmd2.weigelworld.org

See also the references Schwab, Palatnik et al. 2005; and Schwab, Ossowski et al. 2006.
How can I detect translational inhibition caused by amiRNAs?
It is recommended to track expression changes of target genes via RT-QPCR using primers covering the amiRNA cleavage site.

For alternate methods of detecting translational inhibition caused by amiRNAs, please visit the following link: http://wmd2.weigelworld.org

TROUBLESHOOTING
For help with your constructs, please email technical support at info@openbiosystems.com with your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

REFERENCES


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Page 11

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